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Genetic landscape of sporadic unilateral adrenocortical adenomas without *PRKACA* p.Leu206Arg mutation

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Abstract

Context: adrenocortical adenomas (ACAs) are among the most frequent human neoplasias. Genetic alterations affecting the cAMP/PKA signaling pathway are common in cortisol-producing ACAs, while activating mutations in the gene encoding β-catenin (CTNNB1) have been reported in a subset of both benign and malignant adrenocortical tumors. However, the molecular pathogenesis of most ACAs is still largely unclear.

Objective: aim of the study was to define the genetic landscape of sporadic unilateral ACAs.

Design and setting: next-generation whole-exome sequencing was performed on fresh-frozen tumor samples and corresponding normal tissue samples.

Patients: 99 patients with ACAs (74 cortisol-producing and 25 endocrine inactive) negative for p.Leu206Arg PRKACA mutation.

Main outcome measures: identification of known and/or new genetic alterations potentially involved in adrenocortical tumorigenesis and autonomous hormone secretion, genotype-phenotype correlation.

Results: 706 somatic protein-altering mutations were detected in 88/99 tumors (median: 6 per tumor). We identified several mutations in genes of the cAMP/PKA pathway, including three novel mutations in PRKACA, associated with female sex and Cushing's syndrome. We also found genetic alterations in different genes involved in the Wnt/β-catenin pathway, associated with larger tumors and endocrine inactivity, and, notably, in many genes of the Ca2+-signaling pathway. Finally, by comparison of our genetic data with those available in the literature, we describe a comprehensive genetic landscape of unilateral ACAs.

Conclusions: This study provides the largest sequencing effort on ACAs up to now. We thereby identified somatic alterations affecting known and novel pathways potentially involved in adrenal tumorigenesis.

Introduction

Adrenocortical adenomas (ACAs) are among the most frequent human neoplasias with a prevalence of 2-3% in the general population. They are endocrine inactive in 70% of cases, mostly incidentally-discovered, or associated with autonomous cortisol or aldosterone secretion. The genetic basis of several adrenal disorders has been elucidated over the last years following classical genetic approaches and utilizing next-generation sequencing techniques. In particular, the cAMP/protein kinase A (PKA) pathway plays a central role in adrenocortical growth and steroidogenesis. Specifically, genetic alterations affecting the cAMP/PKA pathway, such as germline or somatic mutations in genes encoding the regulatory subunit 1 α of PKA (*PRKAR1A*), the protein Gsα (*GNAS*), and the phosphodiesterases 11A and 8B (*PDE11A* and *PDE8B*) have been reported in cortisol-producing ACAs (CPA) and bilateral micronodular adrenal hyperplasias (1-5).

Recently, we and others have found somatic mutations in the gene encoding the catalytic subunit α of PKA (*PRKACA*) in 35-70% of unilateral ACAs associated with Cushing's syndrome (6-10). These mutations translate into a constitutive activation of PKA by interfering with binding between its regulatory and catalytic subunits (11). Activating mutations in the gene encoding β-catenin (*CTNNB1*) represent another important contributor of adrenocortical growth. At variance with mutations in *PRKACA*, *CTNNB1* mutations had been reported in both adrenocortical adenomas and carcinomas with similar prevalence (10-30%) (12-14), and had been most frequently observed in non-cortisol-secreting tumors (15). Moreover, by using SNP array profiling, we have identified the presence of several recurrent copy number alterations (CNA) in specific chromosomal regions that may also play a role in the pathogenesis of these tumors (16-17).

Despite these recent advances, the pathogenesis of a large proportion of ACAs has remained elusive. In particular, despite representing the most frequent subtype, endocrine inactive adenomas are the least thoroughly investigated, due to their infrequent surgical treatment and thus underrepresentation in tissue based studies. Therefore, the aim of the current study was to define the genetic landscape of sporadic unilateral ACAs by next-generation whole-exome sequencing (WES). In particular, we intended to clarify the molecular mechanisms involved in adrenocortical tumor development and provide genotype-phenotype correlation studies.

Methods

Tissue samples, patients, and clinical annotations

Fresh-frozen ACA tissues (n=99) and corresponding blood or normal adrenal tissues were included from 11 centers belonging to the European Network for the Study of Adrenocortical Tumors (ENSAT, www.ensat.org). Only histologically confirmed unilateral ACAs were included (18). We selected endocrine inactive ACAs (EIA) and CPA without known p.Leu206Arg *PRKACA* mutation (6-10). A subgroup of patients (n=42) had been included in an earlier report (8). All patients provided written informed consent and the study was approved by the ethics committee of each participating institution.

Clinical and hormonal data were collected through the ENSAT registry (https://registry.ensat.org/). Overt Cushing's syndrome (CS) and subclinical CS (SCS) were diagnosed according to current guidelines (19) and defined as previously reported (6). The final series consisted of 74 CPA (39 CS and 35 SCS) and 25 EIA ***(Table 1)***.

A comparative analysis was performed with data available from previous WES studies on CPAs (n=79) (6, 7, 12, 13) and ACC (n=176) (12, 20), and from "The Cancer Genome Atlas" project (21, https://tcga.data.nci.nih.gov/tcga/tcgaCancerDetails.jsp).

WES and data analysis

DNA was extracted from fresh-frozen tissues and checked for signs of degradation as previously described (6). Exomes were enriched in solution and indexed with the SureSelect XT Human All Exon (50Mb kit, version 5, Agilent Technologies, Santa Clara, CA, USA) for library preparation. Sequencing was performed as paired-end reads of 100 bp on HiSeq2500 systems (Illumina, San Diego, CA, USA). Pools of 12 indexed libraries were sequenced on four lanes to an average depth of coverage between 82x and 170x. Image analysis and base calling were performed with Real-Time Analysis software (Illumina). Reads were aligned against the human assembly hg19 (GRCh37) using the Burrows-Wheeler Aligner tool (BWA, v 0.7.5a). Moreover, we performed single-nucleotide variant and small insertion and deletion (indel) calling specifically for the regions targeted by the exome enrichment kit, using SAMtools (v 0.1.19). Subsequently the variants were filtered using the SAMtools varFilter script using default parameters, with the exception of the maximum read depth

parameter, which we set to 9999. Variant detection was done as described earlier (6). In brief, to reduce false positives we filtered out variants that were already present in our in-house database (currently 8,000 exomes) or had variant quality less than 40. Raw read data of the remaining variants are then manually investigated using the Integrative Genomics Viewer (IGV). The frequency of each mutated allele was then evaluated in large population genomics projects, such as the EXAC (Broad) and the "1000 Genomes AF (allele frequency)" data set (Supplementary Table 1).

The Gene Set Enrichment Analysis software (MSigDB database v5.0) (22) was used to identify enriched gene ontology (GO) terms in ranked lists of genes and to perform gene family and pathway analysis (1330 gene sets), including the KEGG (Kyoto Encyclopedia of Genes and Genomes) and the REACTOME pathway (v55) databases.

In silico analysis

Somatic variants were evaluated by both Polymorphism Phenotyping v2 algorithm tool (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2) (23) and SIFT (Sorting Tolerant From Intolerant) algorithm (http://sift.jcvi.org/index.html) (24) to predict the possible impact of an amino acid substitution on the structure and function of a human protein. The variants were classified as possibly pathogenic according to the given thresholds (Supplementary Table 1). Most interesting recurrent genetic alterations were evaluated by *in silico* analysis to predict whether the variants may be damaging. Structural images were prepared with PyMOL software (www.pymol.org). The 3D structures of the mammalian PKA holoenzyme containing catalytic subunit α and regulatory subunit 2 β (PRKACA-PRKAR2B), the stimulatory G-protein α subunit (GNAS, isophorm 15), and the ryanodine receptor RYR1 were acquired from Protein Data Bank (http://www.rcsb.org/pdb/, entries 3TNP, 1AZS, and 4UWA, respectively). Aminoacid changes induced by mutations were identified and displayed using the Chimera v1.10 Software.

Copy number alterations

We compared the results of WES in the present study with previously published CNA data by SNP array profiling (17) available in 14/99 patients.

Transcriptome analysis

Transcriptome analysis was performed by Affymetrix HGU133Plus2, as previously described (25), on an independent cohort of 41 ACAs, including 11 EIAs and 30 CPAs (20 CS and 10 SCS). Targeted next-generation sequencing (AmpliSeq design, IonTorrent sequencing) for *CTNNB1* (Ser45 hotspot, exons 7 and 8), *PRKACA* (L206 hotspot), *GNAS* (R201 hotspot), *PRKACB* and *PRKAR1A* was performed on 37/41 ACAs. Reads were aligned using the human genome assembly hg19 (GRCh37) and variant calling was performed using Torrent Suite Software (v. 4.2.1). Variants were annotated by ANNOVAR package (March, 22nd 2015 release). Variants were visually validated by IGV. Mutations were validated by Sanger sequencing. The mutation status for *CTNNB1* was not available for one ACA, whereas the one for *PRKAR1A* and *PRKACB* was not available in four ACAs.

Transcriptome data were analyzed in R (https://cran.r-project.org/). Unsupervised hierarchical clustering was performed using hclust based on the top 1000 variable transcripts. Differential gene expression was generated with Limma (Linear Models for Microarray Data (26)) R package, using Benjamini-Hochberg correction to adjust p-values. An extensive list of calcium-signaling related genes was provided by the KEGG “Calcium Signaling Pathway” gene list. Enrichment in these calcium genes was sought among the differentially expressed genes, using the Fisher exact test.

Statistical analysis

Unsupervised complete linkage clustering was performed on the rows and columns using the Hamming distance as a similarity metric, to investigate interdependency among genetic alterations. The Fisher’s exact or Chi-square tests, and Mann-Whitney U test were used to investigate dichotomic and continuous variables, where appropriate. Kruskal-Wallis test, followed by Bonferroni *post-hoc* test, was performed for comparison among groups for non-normally distributed variables. Data are shown as median and ranges, if not otherwise specified. Statistical analyses were made using GraphPad Prism (version 5.0, La Jolla, CA, USA) and SPSS Software (version 21, SPSS Inc., Chicago, IL, USA). P values <0.05 were considered as statistically significant.

Results

Overview of genetic findings

Clinical and hormonal characteristics together with the genetic data of patients are provided in ***Table 1.*** We identified 706 non-synonymous protein-altering somatic mutations in 88/99 samples. In 11 tumors no mutations were detected. The somatic variants included 597 missense, 45 nonsense, 31 frameshift, 24 direct splicing, and 9 indel alterations, resulting in a median of 6 somatic mutations in exonic regions per tumor (range: 0-55) (***Figure 1*** and ***Table 1***). According to the PolyPhen-2 algorithm, 203 mutations were classified as probably damaging, 116 as possibly damaging, 271 as benign, and 116 remained undefined. The most frequent substitutions were the C:G>T:A transition and the C:G>A:T transversion (29% and 28% of cases, respectively, ***Supplementary Figure 1***). The complete list of somatic mutations including all the information about the type and localization of genetic alterations, the frequency of the variants in different available databases and the pathogenic classification is summarized in ***Supplementary Table 1***.

Specific genetic alterations

Recurrent somatic mutations (n=56) are shown in ***Table 2***. The most frequent alterations were missense mutations at *CTNNB1*, in a hot-spot region encoding serine in position 45 (n=39). *CTNNB1* mutations occurred in 7/39 patients (18%) with CS, 19/35 subjects (54%) with SCS, and 13/25 patients (52%) with EIA. Moreover, alterations in genes encoding several members of the cadherin superfamily were identified, but only those occurring in *PCDHGA6* were found in at least two samples.

GNAS somatic mutations were identified in 8/74 patients with CPAs (11%), two of them with SCS and six with CS, but in none of the EIAs. In seven patients known activating mutations were found at codon 201, whereas in one patient with CS a novel probably damaging mutation was observed (76A>C, p.Lys58Gln). The 3D *in silico* analysis showed that lysine 58 is near the critical position 201, suggesting a functional significance for p.Lys58Gln substitution, similar to the known GNAS activating mutations (***Supplementary Figure 2***).

Interestingly, we found three novel somatic mutations in *PRKACA* in three patients with CS (p.Trp197Arg, p.245_248.del and p.Glu32Val). Although those mutations occurred outside the known hot-spot region of *PRKACA* in exon 7, the 3D *in silico* analysis pointed towards a potential pathogenic role for two of them. p.Trp197Arg mutation is located at the interface between the catalytic and the regulatory subunit. The exchange of the hydrophobic tryptophan with the hydrophilic, positively charged arginine might lead to alteration in the interaction between the subunits. Moreover, the p.245_248.del affects a region of the catalytic subunit of PKA at the interface with the regulatory subunit, likely inducing a modification that alters the binding of the regulatory to the catalytic subunit. In contrast, the mutation p.Glu32Val, with a hydrophilic, negatively charged glutamate replaced by a hydrophobic valine, is situated outside the interaction region (***Figure 2***).

Several alterations were found in different ryanodine receptors, and those occurring in RYR1 and RYR3 were recurrent. The 3D *in silico* analysis revealed that mutations in *RYR1* (p.Arg1469Gly and p.Val3218Leu) and *RYR2* (p.Lys2264Asn) were located in the clamp regions of the cytoplasmic assembly, while the mutation in *RYR3* (del4516) was pinpointed in the sliding helix between transmembrane and cytoplasmic assemblies (***Supplementary Figure 3***).

Finally, different potentially relevant "private" mutations were detected, including alterations in genes encoding ionotropic (*GRIA1, GRIA2, GRID1, GRIK2, GRIN1, GRIN3B, GRIP1*) and metabotropic glutamate receptors (*GRM3, GRM4, GRM6*). Moreover, a missense mutation in *ARMC5* (p.Pro866Leu) was observed in a 22-mm unilateral left adenoma associated with CS. However, no second hit at the *ARMC5* gene was observed in this tumor. Finally, a probably damaging frameshift mutation (532_533insG) at *TP53* was detected in a 40-mm, endocrine inactive, oncocytic adenoma. Unfortunately, no follow-up data were available to ascertain the clinical course of this patient during the post-operative period.

Gene enrichment and pathway analysis

The gene enrichment analysis identified 605/706 (86%) mutated genes associated with GO terms. Interestingly, Ca^{2+}-signaling, collagen formation, and extracellular matrix organization were recognized as the most significantly represented pathways (***Supplementary Table 2***). The gene family

analysis further showed that eight cytokines and growth factors, 60 transcription factors, including *ATRX* and *MED12*, 16 protein kinases, including *PRKACA*, 14 oncogenes, including *CREB1*, *CREBBP*, *CTNNB1*, and *GNAS*, and four tumor suppressor genes, including *APC* and *TP53* were included among the mutated genes. None of them were mutated in more than one sample (***Supplementary Table 3***).

Genotype-phenotype correlation and transcriptome analysis

No statistically significant relationship was found between the mutation frequency and clinical data (sex, age, tumor size, and cortisol secretion pattern). We classified patients into three groups according to the known or potential biological consequences of the most frequent mutations: subjects with mutations in genes encoding components of the classic Wnt/β-catenin pathway (*CTNNB1*, *APC*, *APC2*, *PCDH15*, *PCDHA8*, *PCDHB11*, *PCDHA10*, *PKP2*), those with alterations in genes encoding components of the cAMP/PKA pathway (*GNAS*, *PRKACA*, *PRKAR1A*, *CREB1*, *CREBBP*, *ADCY3*, *GRM3*, *GRM4*, *GRM6*), and those with mutations in genes encoding components of Ca^{2+}-dependent signaling (*CACNA1C*, *CACNA1E*, *CACNG8*, *RYR1*, *RYR2*, *RYR3*, *GRIA1*, *GRID1*, *GRIK2*, *GRIN1*, *GRIN3B*, *GRIP1*) (***Supplementary Table 4***). The results of the unsupervised binary clustering analysis and the relationship between the genetic landscape of tumors and the clinical phenotype of the three groups of patients are shown in ***Figure 3A*** and ***Supplementary Table 5***. Patients with mutations in genes encoding components of the Wnt/β-catenin pathway were older, had larger tumors, and carried a higher total number of mutations than those without these aberrations ($P<0.05$). In contrast, patients with mutations in the genes encoding component of the cAMP/PKA pathway were more frequently female and younger, in comparison to subjects not carrying mutations ($P<0.01$). Mutations in genes encoding components of Ca^{2+}-dependent signaling were associated with a higher number of mutations when compared to those without ($P=0.001$), whereas no difference in clinical and hormonal parameters was evident.

The results of the unsupervised clustering according to the results of the transcriptome analysis are shown in ***Figure 3B*** and ***C***. After considering the expression level, transcriptome profile could clearly identify four groups and well separated patients with CS from those with EIA and SCS,

and tumors with mutations of the cAMP/PKA pathway from those with mutations in the Wnt/β-catenin or without mutations in one of those two pathways, showing significant enrichments in calcium-related genes (***Figure 3B***). Surprisingly, restricting the analysis only to genes of the Ca^{2+} signaling pathway, the transcriptome profile was also able to clearly divide the patients in four groups. The four clusters showed a good separation in patients with CS *vs* those with EIA or SCS, as well as tumors with mutations in the cAMP/PKA *vs* Wnt/β-catenin pathway (***Figure 3C***).

Combined genetic and genomic analysis

We further analyzed current WES data in combination with those from SNP array profiling available for a subgroup of 14/99 ACAs (three with CS, seven with SCS, and seven with EIA) (17). As summarized in ***Table 3***, some large chromosomal regions (16p13.3-13.2, 19p13.3-12, 7p22.3-22.1, 11p15.5, 20q13.3) and several genes were affected by recurrent CN gains, including genes involved in Wnt/β-catenin (*APC2* in two samples), cAMP/PKA pathways (*PRKACA, PRKR1B, AKAP8* in two samples) or Ca^{2+}-dependent signaling (*CACNA1H* in five samples, *CACNA1A* and *CACNA1B* in two samples). There was no significant difference in total number of CNA between tumors with or without somatic mutations.

In 4/14 tumors no somatic mutations were detected by WES. One of those (CS) showed a large amplification at 19p13.2-12 including the genes *AKAP8, CACNA1A, PDE4C* and *PRKACA*. The second tumor (SCS) had amplifications at 7p22.2, which included *PRKAR1B* and 16p13.3. The third sample (EIA), presented a CN gain at chr11p15.5 and several micro-amplifications, whereas the last one (SCS), did not show any CNA in regions or genes with presumed functional relevance.

Systematic review of genetic data available in unilateral adrenocortical tumors

We compared the genetic findings of the present analysis with WES data available in the literature for ACA (n=69 CPA) and ACC (n=176) (***Supplementary Table 6***). The analysis of *PRKACA* wild-type benign tumors (n=94 CPA+25 EIAs) showed that mutations in genes involved in cAMP/PKA pathway were present only in CPA (20% of cases), whereas alterations of genes involved in Wnt/β-catenin

signaling were mutated in 49% of CPA and in 76% of EIAs. Alterations in genes involved in Ca^{2+}-dependent signaling were found in 14% of CPA and in 16% of EIA.

We performed an unsupervised clustering with all WES data available for ACAs (n=168), subdividing the mutations according to the three groups defined above (***Supplementary Figure 4***). We also performed a canonical pathway analysis considering all the 168 ACA samples together and subdividing them into the three groups (49 CPA with *PRKACA* mutations, 94 CPA without *PRKACA* mutations and 25 EIA, ***Supplementary Table 7*****).** In brief, genes involved in the "cancer pathways" were present in all groups, while genes of the "calcium signaling pathway", "collagen formation" or "ECM organization" were not recorded among the *PRKACA* mutated CPAs.

Finally, we observed that 23% of somatic mutations observed in our cohort were previously reported in at least one of the 176 ACC samples and 6% in at least two ACCs (***Supplementary Table 6*** and ***Supplementary Figure 5)***. As expected, mutations in *CTNNB1*, the most frequent alterations, were detected in 15% of ACC and in 25% of ACA (34% of *PRKACA* wild-type CPA and 52% of EIA). Interestingly, mutations in different members of proto-cadherin family were frequently observed in 13% of CPA negative for *PRKACA* mutations, 24% of EIA and 15% of ACC.

Discussion

The present study represents the most comprehensive genetic characterization of unilateral ACA. In this large European series we analyzed also for the first time endocrine inactive adenomas that represent the most frequent but less investigated type of ACAs. By restricting the investigation to patients without mutations in the predominant hot-spot region of *PRKACA* (p.Leu206Arg), WES analysis highlights substantial heterogeneity of the genetic background of cortisol-producing and endocrine inactive ACAs, and separates well those tumors from aldosterone-producing ACA (27, 28). Overall, we identified 706 somatic mutations with a median of 6 per tumor. Many of the 605 mutated genes encoded components of the cAMP/PKA, the Wnt/β-catenin or, more surprisingly, the Ca^{2+}-dependent signaling pathway.

Among genetic alterations of the cAMP/PKA pathway, *GNAS* somatic mutations were the most frequent, being associated with cortisol production, accordingly with published data (7, 9-10). In

addition to the previously reported hot-spot mutations, a novel substitution p.Lys58Gln was found in a patient with CS with potential functional relevance in our *in silico* model. Likewise, three novel somatic mutations in *PRKACA* were detected in three CPA associated with CS. Interestingly, *in silico* data provide evidence that the p.Trp197Arg substitution and the p.245-248 deletion may be able to alter the interaction between the catalytic and the regulatory subunit of PKA, similarly to what described for the p.Leu206Arg mutation (11). Moreover, the essential role of the phosphorylation site Trp197 in the binding to PKA regulatory subunit was already described in 1997 (29). In contrast, the localization of the mutation p.Glu32Val outside known interacting regions of the catalytic subunit, do not allow any speculation on the biological relevance of this substitution. Other mutated components of the cAMP pathway included *PRKAR1A, CREB1* (cAMP responsive element binding protein), *CREBBP* (CREB binding protein) and three genes encoding metabotropic glutamate receptors (mGluRs, *GRM3, GRM4, GRM6*). The mutated mGluRs in our cohort belong to the group II and III mGluRs, which are G-protein-coupled receptors involved in regulation of intracellular cAMP levels. Interestingly, mGluR3 has been previously suggested to be involved in the regulation of steroidogenesis in adrenocortical tissues (30). Considering the relationship with the clinical data, mutations in component of the cAMP/PKA pathway occur invariably in young patients with cortisol-secreting tumors. Those results are in line with the data previously published by our group (6, 8) and others (7, 9-10), confirming that additional alterations of the cAMP pathway, apart from the well-known *PRKACA* mutations, are associated with a severe hormonal phenotype and, likely, early diagnosis.

Among mutations affecting genes of the Wnt/β-catenin pathway, as expected, the most common were somatic mutations in *CTNNB1* (39% of cases). They occurred more frequently in patients with SCS and EIA (54% and 52% of cases, respectively) than in those with CS (18%), as previously reported (15). These findings may further confirm a predominant role of *CTNNB1* mutations in early adrenocortical tumorigenesis. Among the components of the Wnt/β-catenin pathway, genes encoding for the plakophilin (*PKP2),* member of the arm-repeat (armadillo) gene family, the adenomatosis polyposis coli (*APC*) and *APC2*, and four members of the protocadherin family (*PCDH15, PCDHA8, PCDHA10, PCDHB11)* were recognized. Protocadherins play a major

role in cell-cell adhesion and interfere with the β catenin signaling proliferation pathway (31). Some members of the protocadherin family have recently been recognized as candidate tumor suppressor genes (31), and somatic mutations have been reported in squamous cell carcinoma, colon adenocarcinoma and melanoma (see COSMIC, http://cancer.sanger.ac.uk/cosmic/gene/analysis). Moreover, protocadherins may play a role in cell-cell adhesion and interfere with the Wnt/β-catenin signaling pathway (32), supporting the hypothesis that alterations of this Wnt/β-catenin regulatory signal may be relevant for adrenocortical tumorigenesis. In this context, it is important to mention that the regulator of Wnt/β-catenin pathway *ZNRF3*, recently reported as one of the most frequently altered genes in ACC (15), was not identified among mutated genes in our ACA series. In general and similarly to what previously reported for *CTNNB1* mutations, the genetic alterations in components of the Wnt/β-catenin pathway were mostly found in older patients with larger and inactive tumors (19).

Among Ca^{2+}-dependent signaling pathways, genes encoding Ca^{2+} receptors (*CACNA1C* and *CACNA1E)*, ryanodine receptors (RyRs), ionotropic glutamate receptors (iGluRs) and one glutamate receptor interacting protein (*GRIP1)* were included. The RyRs are intracellular Ca^{2+}-release channels found on the sarcoplasmic reticulum of myocytes and on the endoplasmic reticulum of several non-muscular organs (33). There is some evidence on the potential role of RyR alterations on adrenal function (34). According to our *in silico* analysis of *RYR1* and *RYR2* mutations and considering that the interaction between transmembrane and cytoplasmic domains of those receptors is an important mechanism in Ca^{2+} release modulation (35), it is well conceivable that the mutations found in our cohort may be biologically relevant. Several genes responsible for regulation of intracellular Ca^{2+} levels are known or suspected to be involved in the pathogenesis of endocrine tumors, such as aldosterone-producing adenomas (*KCJN5, ATP1A1, ATP2B3*, and *CACNA1D*) (27, 28) and GH-secreting pituitary adenomas (36, 37). In contrast, the role of alterations of Ca^{2+} signaling in the pathogenesis of CPA is not well understood, even though it has been demonstrated that adrenal fasciculata cells express high levels of T-type and L-type Ca^{2+} channels that may regulate cortisol secretion (38). Additionally, Ca^{2+} channels could be involved in molecular mechanisms of apoptosis regulation and cancer transformation (39), leading us to speculate on the proliferative role of this pathway in adrenocortical cells. Interestingly, the transcriptome analysis performed on our

independent cohort clearly showed that the expression of Ca^{2+} signaling-related genes in ACAs not associated with primary hyperaldosteronism is able to classify patients into meaningful clusters. In fact, the unsupervised clustering restricted to the expression levels of those genes, provided a good separation of patients with CS from those with SCS and EIA, and tumors with mutations in the cAMP-PKA pathway from those with Wnt/β catenin alterations. This finding, together with the identification of somatic mutations in Ca^{2+} signaling genes in our study, provides indirect evidence for a role of Ca^{2+}-related pathways in the tumorigenesis and steroidogenesis of non-aldosterone secreting ACAs. Further studies will be necessary to unravel the specific underlying mechanisms.

Additional insights come from the combined analysis with CNA available from a previous SNP array profiling (17) in a well representative subgroup of present ACAs (three CS, seven SCS, and seven EIA), including four samples without any somatic mutations, four with mutations in the Wnt/β catenin pathway, four with mutations in the cAMP/PKA pathway and two samples without known driver mutations. Here, we observed amplifications in several components of the Wnt/β-catenin, cAMP/PKA or Ca^{2+}-dependent signaling pathways. While this provides additional evidence for a major role in the pathogenesis of ACA, no differences were observed between ACA with or without somatic mutations.

According to the results of the pathway analysis, components of Ca^{2+} signaling, collagen formation, and extracellular matrix organization were among the most significantly represented. Extracellular matrices (ECM) are secreted molecules composed of glycoproteins, collagens, glycosaminoglycans and proteoglycans that can regulate cell migration, differentiation, proliferation and survival by communicating with intracellular cytoskeleton and growth factor signals (40). Interestingly, a putative role for ECM expression has been hypothesized in the development of human adrenal cortex (41). Moreover, a previous transcriptome study on ACAs identified enrichment in genes related to ECM (42). However, we observed only “private” mutations in ECM and collagen formation pathways and it is unclear whether they derive from proliferative processes or might represent early events in adrenocortical tumorigenesis.

We also performed an unsupervised clustering considering the WES data available for all ACA together (n=168) and separated for CPAs with or without *PRKACA* mutations (n=49 and 94,

respectively), providing results similar to that obtained in our present series (***Supplementary Figure 4***). In addition, in this very large series, we observed that most genetic alterations in the cAMP/PKA signaling pathway were not associated with alterations at the Wnt/β-catenin or Ca^{2+}-dependent signaling pathway, further confirming their major role in the pathogenesis of CPAs.

The analysis of the genetic landscape of ACAs and ACCs provides indirect evidence for the existence of an adenoma-carcinoma sequence in adrenocortical tumors. For instance, the frequent C:G>T:A transitions observed in our patients has been found to be a feature of most cancer types (43), including ACC (12). Moreover, 6% of somatic mutations identified in our series were previously observed in at least two ACC samples (12, 20-21), giving support to a potential role of early genetic alterations in a multistep malignant transformation process. In this context, recurrent mutations in the hot-spot region of *CTNNB1*, were among the most commonly observed alterations in ACA and ACC. Thus, it is tempting to speculate that an adenoma-to-carcinoma multistep progression might occur in a subset of adrenocortical tumors bearing *CTNNB1* mutations, with β-catenin activating mutations as an early step in adrenocortical tumorigenesis. In sharp contrast, 11/99 tumors did not show any detectable genetic alteration by exome-sequencing. This finding might be due to limitation of the WES technique or to the pathogenesis of some ACA, which should be further evaluated for different genetic aberrations (alterations in intronic regions, alternative splicing, or gene fusions).

One limitation of the current study is the lack of functional data so that we can only speculate on the biological role of newly identified genetic variants. However, also due to the large number of "private" mutations, this was beyond the scope of this report that was focused on providing a comprehensive overview of acquired genetic findings and potential genotype/phenotype correlations. Thus, targeted functional experiments will be required to characterize mutations not described in the literature. In contrast, the large samples size, including for the first time also endocrine inactive adenomas, with detailed clinical characterization and the integration of previous WES data available for cortisol-secreting adenomas and carcinomas are relevant strengths of this collaborative project.

In summary, our study represents the largest sequencing effort on sporadic unilateral adrenocortical adenomas and demonstrates the heterogeneity of the genetic background of ACAs without *PRKACA* p.Leu206Arg mutation. Apart from the known somatic mutations, no other recurrent

mutation can alone explain the processes that lead to tumor formation and hormone hypersecretion. However, the provided landscape and the genetic alterations in newly described pathways (i.e. Ca^{2+}-dependent signaling) are shedding light on the pathogenesis of adrenocortical tumors and are providing a solid basis for future molecular analysis.

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Figure legends

Figure 1. Total number of somatic mutations in each adrenocortical adenoma (n=99) evaluated by next generation exome sequencing (median: 6 mutations per tumor).

Figure 2. *In silico* analysis of the 3D structure changes of three novel somatic mutations in *PRKACA* gene (589A->G, p.Trp197Arg; 95T->A, p.Glu32Val; and deletion in position 731-745, p.245-248). a) wild type; b) the p.Trp197Arg mutation is at the interface between the catalytic and regulatory subunit. The exchange of the hydrophobic tryptophan with the hydrophilic, positively charged arginine leads to changes in this interaction. The p.245_248.del also affects a region of the catalytic subunit of PKA at the interface with the regulatory subunit. The deletion of this region probably leads to modification of the 3D structure and affects the binding of the regulatory to the catalytic subunit. The mutation p.Glu32Val is situated outside the interaction region between the catalytic and regulatory subunits of PKA or any other reported interaction region of catalytic subunit of PKA.

Figure 3. A. Heat map of the most recurrent somatic mutations classified according to their known or potential biological consequences: mutations in genes encoding components of Wnt-β catenin pathway, those in genes encoding members of the cAMP/PKA pathway, and mutations in genes involved in Ca^{2+}-signaling (n=99 samples). The relationship with the total number of somatic mutations and clinical parameters is also shown. **B and C.** Transcriptome analysis of the cohort of additional 41 adenomas. The unsupervised clustering performed according to the expression level from whole transcriptome profiling is shown in **B**, whereas the clustering restricted to Ca^{2+} signaling-related genes is shown in **C**. The relationship with somatic mutations and clinical parameters, as well as the heat map of under-/over-expressed genes in the two pathways is also shown.